Neuron, Vol. 20, 667–681, April, 1998, Copyright ©1998 by Cell Press

A Role for Abl in Notch Signaling

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Summary

Abl is an axonal tyrosine kinase that has yet to be clearly linked to a receptor; Notch is a receptor for which the signaling pathway remains incompletely understood. We show here that *Notch* and *abl* mutations interact synergistically to produce synthetic lethality and defects in axon extension. Surprisingly, we cannot account for these axonal aberrations on the basis of changes in cell identity. We show, moreover, that Notch is present in the growth cones of extending axons, and that the Abl accessory protein Disabled binds to a signaling domain of Notch in vitro. We therefore speculate that Disabled and Abl may play a role in Notch signaling in *Drosophila* axons, perhaps by binding to the Notch intracellular domain.

Introduction

Notch is a transmembrane receptor that is required for the proper development of a wide variety of cell types during *Drosophila* embryogenesis (Shellenbarger and Mohler, 1978; Fortini and Artavanis-Tsakonas, 1994). Mutations in the *Notch* gene lead to severe defects in cell differentiation and morphogenesis in the nervous sytem, musculature, gonads, and many other tissues. Recent work has suggested the following model for the molecular mechanism by which Notch regulates the segregation of neural precursors (Artavanis-Tsakonis et al., 1995). In the absence of extracellular signals, the transcription factor Su(H) is tethered to the intracellular domain of Notch. When Notch is stimulated by its ligand, Delta, Su(H) protein becomes activated by a process that requires Deltex, another Notch-binding protein. Activated Su(H) then translocates to the nucleus and binds to the promoters of the *E(spl)* genes, stimulating their transcription and in turn regulating neurogenesis. Equivalent mechanisms are thought to act in other tissues where Notch functions and in signaling by Notch homologs in other metazoans; however, the mechanism by which Su(H) protein is activated remains obscure (Artavanis-Tsakonis et al., 1995; Wettstein et al., 1997). Moreover, some experiments suggest the existence of other, Su(H)-independent signaling pathways acting downstream of Notch in some developmental contexts (Lecourtois and Schweisguth, 1995; Shawber et al., 1996; Wang et al., 1997). We therefore set out to ask what other signal transduction proteins might act in the *Notch* pathway.

Many transmembrane receptors that regulate differentiation and morphogenesis do so by the activation of protein kinase cascades, often including protein tyrosine kinases (Schlessinger and Ullrich, 1992; Taniguchi, 1995). We therefore examined whether a protein tyrosine kinase might be involved in Notch function. We investigated several tyrosine kinases; among them was D-Abl, the *Drosophila* homolog of the vertebrate *abl* oncogene. Abl is a cytoplasmic tyrosine kinase that is widely expressed, phylogenetically conserved, and thought to be involved in the development of a variety of tissues (Hoffmann, 1991; Schwartzberg et al., 1991; Tybulewicz et al., 1991).

The genetics of *Drosophila abl* have been studied extensively (Hoffmann, 1991). *abl* mutations are semiviable in homozygotes and produce no discernable embryonic defects. A substantial fraction of mutant animals die as pupae, and those that survive to adulthood are born with defects in eye morphology (rough eyes), are largely sterile, and die within a few days after eclosion. It may be that the viability of *abl* null individuals reflects the existence of one or more additional tyrosine kinases in the fly that can substitute for Abl (Simon et al., 1985; Hoffmann, 1991; Takahashi et al., 1996), though other models are also conceivable (Hoffmann, 1991). A genetic requirement for *abl* can be uncovered, however, by reducing the amount or activity of other proteins that normally collaborate with Abl (Gertler et al., 1989). For example, if the dosage of the *disabled* (*dab*) gene is reduced by half, as in a fly with the genotype $dab^{-}/+$; abl^{-}/abl^{-}, then such an individual suffers gross defects in axon extension and dies as an embryo. Consistent with this phenotype, Abl is concentrated in axons (Gertler et al., 1989), as is Dab (Gertler et al., 1993; Howell et al., 1997), and it is thought that these proteins play a direct role in the control of the axonal cytoskeleton (Hoffmann, 1991). Genes that interact synergistically with *abl* are collectively termed *HDA* loci (haploinsufficient, dependent on *abl*; Hoffmann, 1991). Similar synergistic genetic interactions are often found in genes whose products interact directly, such as the different constituents of multiprotein complexes (Bruno et al., 1996). While it has not been shown directly that *HDA* loci encode proteins that associate with Abl, the sequence of Dab makes it a good candidate to bind to the Abl SH2 domain (Gertler et al., 1993; Songyang et al., 1993), and indeed the mouse Dab homolog binds to mouse Abl in vitro and to the closely related SRC SH2 domain in vivo (Howell et al., 1997). Similarly, the *abl*-interacting gene *ena* is thought to encode a direct substrate of the Abl kinase (Gertler et al., 1995).

We show here that *Notch* has the genetic and developmental properties of an *HDA* locus. In the context of an *abl* mutation, modest reduction of *Notch* levels leads to synthetic lethality and defects in axon extension. These axonal defects, moreover, are restricted to axon tracts that are known to require *Notch* for their proper development. Our observation that Notch is present in extending axons and in growth cones, together with the finding that cell identity appears largely to be unaffected by the *Notch/abl* interaction, raises the possibility that Notch and Abl may work together in the axon to promote axon extension. Consistent with this idea, we find that the requirement for *Notch* in axon patterning is genetically

Table 1. Synthetic Lethality of *Notch* and *abl* Mutations

Genotype	% viable
N^{+}; $abl^{1}/Df(3L)abl^{stJ7}$	41%
N^{ts1}; $abl^{1}/Df(3L)abl^{stJ7}$	lethal (<1.5%)
N^{+}; $abl^{2}/Df(3L)abl^{stJ7}$	29%
N^{ts1}; $abl^{2}/Df(3L)abl^{stJ7}$	lethal (<1%)
N^{ts}; $abl^{2}/Df(3L)abl^{stJ7}$ $Tn[abl^{+}]$	78%
$Df(1)N^{8}/N^{+}$; $abl^{2}/Df(3L)abl^{stJ7}$	lethal (<4%)
$Df(1)N^{8}/N^{+}$; $abl^{2}/Df(3L)abl^{stJ7}$ $Tn[abl^{+}]$	95%

Flies of the indicated genotypes were generated by standard genetic crosses. Experimental and control crosses for N^{ts1} (lines 1–5) were raised at 18°C; at this temperature, N^{ts1} by itself is homozygous viable, but its activity is sufficiently reduced to reveal the synthetic genetic interaction between *Notch* and one of its ligands, *Serrate* (E. G., data not shown). In this experiment, crosses involving $Df(1)N^{8}$ were raised at 25°C. Percent viabilities were calculated based on control genotypes in each cross. Data from one typical experiment is shown here. In the table, ''lethal'' indicates that no flies of this genotype were recovered; the upper limit of possible viability (based on this trial) is given by the value in parentheses. Strong *Notch* alleles were scored in heterozygous females; N^{ts1} was scored in hemizygous males.

separable from its function in the control of cell identity. Finally, we find that the Abl accessory protein, Disabled, binds directly to the Notch intracellular domain in vitro and interacts genetically with *Notch* and *abl* in vivo. One potential interpretation of these data is that Notch promotes axon extension by physically recruiting Disabled and possibly Abl.

Results

Synthetic Lethality of *Notch* and *abl* Mutations

The hallmark of *abl*-interacting genes (called *HDA* loci) is the synthetic lethality that ensues when the concentration or activity of an *HDA* gene product is reduced in a homozygous *abl* mutant genetic background (Gertler et al., 1989; Hoffmann, 1991). To ask whether *Notch* is an *HDA* locus, we generated flies bearing one of three unrelated *Notch* alleles together with various heteroallelic combinations of three different *abl* alleles and assayed adult viability.

Reduction of *Notch* activity is lethal in an *abl* mutant background. In a typical experiment (Table 1), we found that heteroallelic combinations of *abl* alleles were 20%–40% viable compared to control genotypes. In contrast, no adult flies were observed that were both trans-heterozygous for a pair of *abl* alleles and either heterozygous for a strong *Notch* allele (at 25°C) or hemizygous for N^{ts1} (at 18°C). The synthetic lethality of *Notch* and *abl* was substantially rescued by introducing an additional wild-type copy of *abl* on a transposon and partially rescued by introducing a genetic duplication bearing *Notch*. Thus, *Notch* acts genetically as an *HDA* locus.

The *Notch/abl* Interaction Does Not Cause Neurogenic or Anti-Myogenic Phenotypes

The observation of a synergistic, lethal genetic interaction between mutations in *Notch* and *abl* suggested that Abl may be an unrecognized element of a Notch signaling pathway. To distinguish whether Abl is involved in all or just a subset of *Notch*-dependent processes, we examined the effect of the *Notch/abl* interaction on neurogenesis and myogenesis. In the absence of *Notch* function, most of the embryonic ectoderm develops as neural tissue (Lehman et al., 1983), while the somatic mesoderm largely fails to form recognizable muscles (Bate et al., 1993). If Abl is involved in all Notch signaling, we should detect such neurogenic and anti-myogenic phenotypes in embryos bearing synthetic lethal combinations of *Notch* and *abl* mutations. We therefore generated animals that were either homozygous for N^{ts1} (at semipermissive temperature) or heterozygous for a strong *Notch* allele and that simultaneously bore a heteroallelic combination of *abl* mutations. We will refer to these as *N/abl* embryos.

N/abl embryos did not display the neurogenic or anti-myogenic phenotypes we would expect if Abl were involved in all Notch signaling. Figure 1A is a ventral view of an *N/abl* embryo stained with mAb 44C11 to label all neuronal nuclei. A morphologically normal ventral nerve cord is apparent in the embryo, as are the ventral clusters of sensory neurons. Similarly, Figure 1B shows a lateral view of an *N/abl* embryo stained with anti-myosin. A wild-type pattern of somatic muscles is clearly visible.

Axonal Defects in *N/abl* Animals

Since the *Notch/abl* interaction does not overtly disturb neurogenesis or myogenesis, we inferred that Abl is probably involved in only a subset of Notch signaling events. While *Notch* is most studied for its control of cell fate, there is a discrete set of axons whose extension requires *Notch* (Giniger et al., 1993a). Specifically, appropriate temperature shifts of a N^{ts} mutant block CNS axons from growing between successive neuromeres and prevent the peripheral intersegmental nerve (ISN) from traversing the lateral portion of the embryo. *abl* is probably involved in the growth of most axons. Since *Notch* and *abl*, separately, are each required for extension of at least some axons, and since we know of no other aspect of embryonic development that requires both of these proteins, we examined axonal development in *N/abl* embryos.

We found that the gross morphology of the nervous system was typically normal in *N/abl* embryos, but that specific axon tracts failed to develop (Figure 2). Axonal defects were observed in all of the nerve tracts that are known to require *Notch*: CNS longitudinal tracts between neuromeres (Figures 2A–2D) and the lateral portion of the ISN (Figures 2E and 2F). In contrast, longitudinal tracts within each neuromere and commissural tracts appeared normal, as did the dorsal and ventral portions of the ISN. The penetrance (fraction of embryos affected) and expressivity (number of affected hemisegments per affected animal) of the *N/abl* axonal phenotype depended on the particular combination of alleles used. For example, in a typical experiment employing the heteroallelic combination abl^{1}/abl^{2}, we found that 95% of embryos that were also $DfN^{8}/+$ showed axonal defects (affected hemisegments/affected embryo: 8.9 CNS and 2.0 PNS), whereas 46% of embryos that were also N^{ts1} showed defects (affected hemisegments/affected embryo: 2.5 CNS and 1.4 PNS, n = 100 stage [st] 15–17

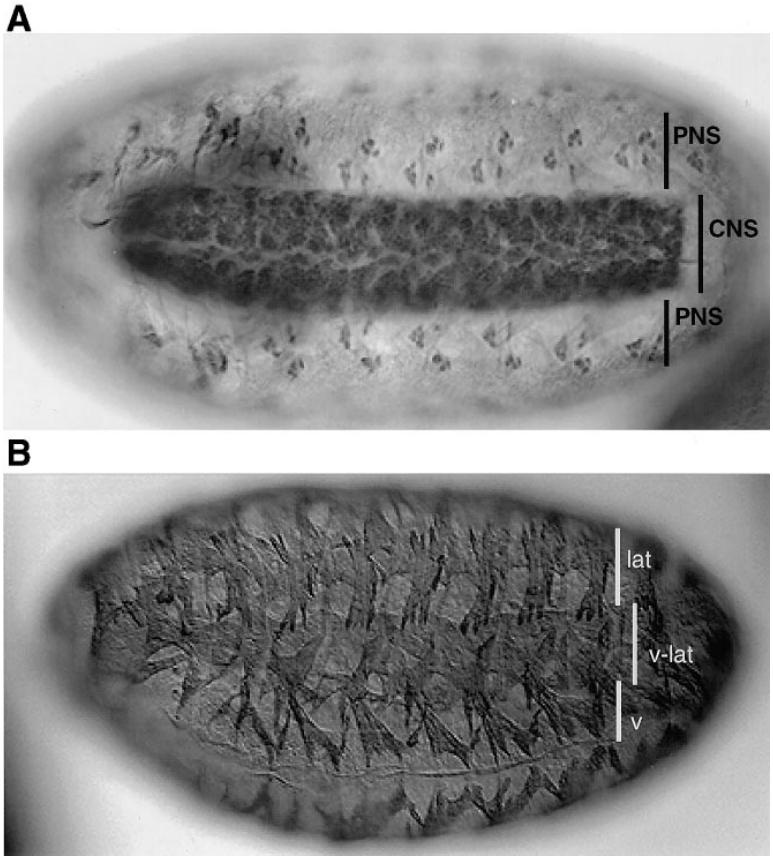

Figure 1. Neurogenesis and Myogenesis in *N/abl* Embryos

Embryos of the genotype N^{ts1};abl^{1} were raised at 25°C to st 16/17, stained with appropriate antibodies to assay neurogenesis and myogenesis, and visualized with peroxidase histochemistry. At this semipermissive temperature, N^{ts1} by itself causes reduced viability but does not produce overt morphological abnormalities in the embryo (compare Figure 2A). (A) Ventral view of an embryo stained with anti-Elav to label all neuronal nuclei. Ventral nerve cord (CNS) is visible, as are ventral clusters of sensory neurons (PNS); all are wild type in appearance. (B) Lateral view of an embryo stained with anti-myosin heavy chain to label somatic muscles. Well-differentiated lateral, ventrolateral, and ventral muscles are clearly apparent in the image. Anterior is to the left in all figures. Dorsal is at the top in (B) and in all figures showing lateral views of embryos.

embryos in each sample). Partial expressivity at a similar level has been observed for other mutations that affect this same pair of axon tracts, such as *lola* (Giniger et al., 1994). The penetrance of axonal aberrations was very low in control genotypes: the fraction of embryos displaying any axonal defects was ~5% for $abl^{1}abl^{2}$ alone and 1%–2% for $DfN^{8/}+$ or for N^{ts1} at 25°C. Introduction of an abl^{+} transposon suppressed *Notch/abl*-dependent axonal defects substantially, though not completely, both in the PNS and in the CNS; this abl^{+} transposon has been characterized previously by Hoffmann and coworkers and is known to be less active than the chromosomal *abl* gene (F. Gertler, personal communication).

Cellular Analysis of Axonal Defects in *N/abl* Embryos

In principle, the axonal defects we observe in mature *N/abl* embryos could reflect a failure either to form axon tracts or to maintain them. Moreover, if the defect is in the initial development of the axon, it could be due to the absence of required substratum cells, the absence or improper identity of the neurons themselves, or else the failure of the actual guidance machinery of the growth cone. To discriminate among these possibilities, we examined directly the development of pioneer neurons and substratum cells for affected axon tracts.

We first analyzed the initial extension of pioneer axons in *N/abl* embryos. Consistent with the terminal phenotype, the combined MP fascicle, the first to form between successive neuromeres (Goodman and Doe, 1993), is obviously aberrant from a very early stage (st 13) (Figures 3B and 3C; compare wild type, Figure 3A). In contrast, both the anterior and posterior commisures appear to develop normally, as do the longitudinal tracts within the neuromeres.

We next examined the substratum cells for affected axon tracts. The MP fascicle projects between neuromeres on a specific Fasciclin II–expressing glial cell, LG5 (Goodman and Doe, 1993), and this was present in affected hemisegments (Figure 3D). In the PNS, the direct cellular substratum for ISN extension in the lateral part of the embryo is a cluster of lateral peritracheal cells that lie along the trachea (Giniger et al., 1994). Examination of stalled motor axons in an *N/abl* embryo shows that the nerve frays and stalls precisely as it attempts to grow along the trachea; the presence of the peritracheal cells can be clearly seen in the Nomarski image of this affected segment (Figure 3E, cellular outlines traced in Figure 3E′).

Since substratum cells for affected axon tracts are present in *N/abl* embryos, we next examined the pioneer neurons themselves. The positions and cell body morphologies of the sensory neurons in the PNS provide sensitive assays for the identities of these cells (Bodmer and Jan, 1987), and these typically appeared wild type (Figure 2F). In the CNS (Goodman and Doe, 1993), the neuron aCC that pioneers the ISN and innervates the most dorsal muscle (muscle 1) is readily apparent in *N/abl* embryos (Figure 3B). The neurons that pioneer

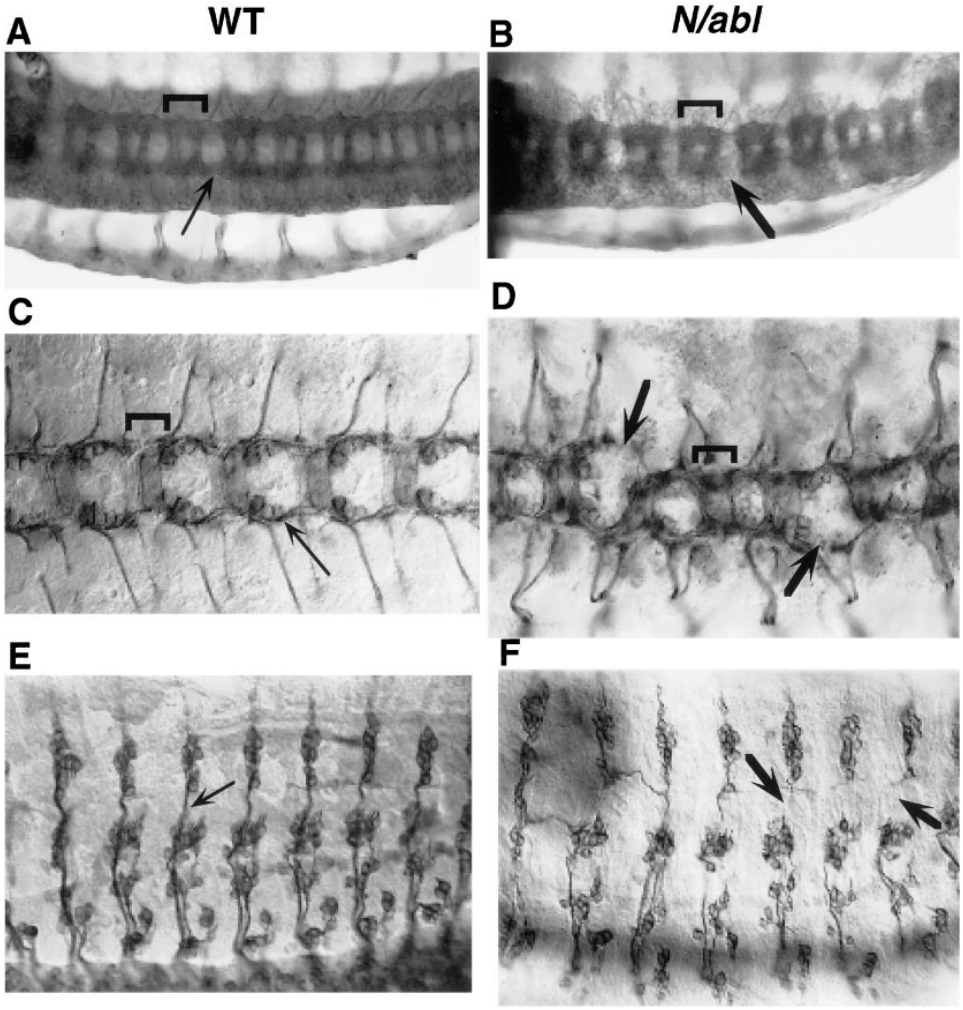

Figure 2. Axonal Defects in Mature *N/abl* Embryos

Wild-type ([A], [C], and [E]) or *N/abl* ([B], [D], and [F]) embryos were raised at 25°C, stained with antibodies to label various axonal processes, and visualized with peroxidase histochemistry. In (B), (D), and (F), thick arrows indicate places where nerves are interrupted in *N/abl* embryos; their wild-type counterparts are indicated by thin arrows in (A), (C), and (E). Defects in (B) and (D) are restricted to the longitudinal tracts between successive neuromeres. In (F), defects are found in the lateral portion of the ISN. Other nerves, and other portions of affected nerves, appear largely wild type. Bracket indicates one neuromere in (A–D).

(A and B) Ventral views of st 16 embryos stained with anti-HRP to label all neurons.

(C and D) Dorsal views of st 15 CNS stained with anti-FasII to label all motoneurons and a subset of interneurons.

(E and F) Lateral views of st 16 embryos stained with mAb 22C10 to label all sensory neurons.

The "wild-type" embryo in (A) is a N^{ts1} embryo, to demonstrate that development at 25°C does not lead to gross morphological aberrations in this genotype; other wild-type embryos are Oregon R. N^{ts1};abl^{-}/abl^{-} embryos raised at 18°C did not display detectable axonal defects (data not shown). Genotypes of *N/abl* embryos are: (B) $Df(1)N^{8}/N^{+}$;abl^{1}/abl^{2} (B) and N^{ts1};abl^{1} ([D] and [F]).

the MP fascicle within the CNS are MP1, pCC, dMP2, and vMP2, and cells whose positions and axonal morphologies are appropriate for these cells can be seen in affected hemisegments of *N/abl* embryos (Figures 3B–3D).

Molecular Analysis of Cell Identities in *N/abl* Embryos

The observation of morphologically normal pioneer neurons and substratum cells in *N/abl* embryos was surprising, since perturbation of cell identity seemed a priori to be the simplest explanation for the axonal defects in these embryos. We therefore investigated molecular markers for the development of affected pioneer neurons to determine whether their identities were disturbed in some more subtle way.

The homeobox proteins Ftz and Eve are expressed in the pioneer neurons aCC and pCC, and changes in the expression of either protein disrupts guidance of some axons (Doe et al., 1988a, 1988b). We found Eve expression to be wild type in these cells in 98% of hemisegments of N^{ts}/*abl* embryos (n = 186 hemisegments; Figure 4B) and >85% of hemisegments of $DfN^{8}/+$;*abl*/*abl* embryos (n = 239). Ftz was wild type in these cells in 98% of hemisegments of N^{ts}/*abl* embryos (n = 145; Figure 4A).

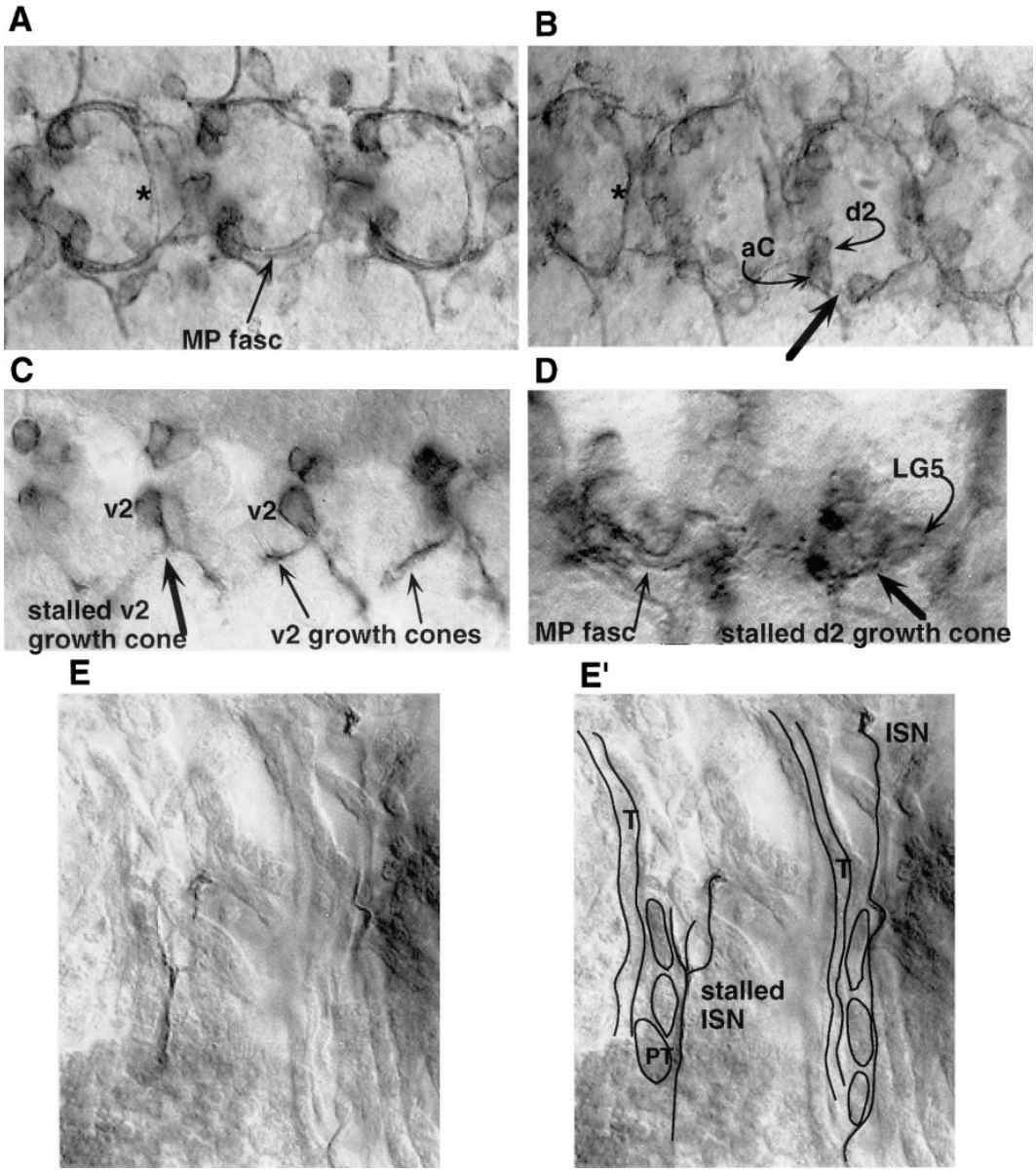

Figure 3. Cellular Analysis of Axonal Defects in *N/abl* Embryos

Wild-type (A) or *N/abl* (B–E) embryos were stained with antibodies to label pioneer neurons and substratum cells for nerves affected by the *N/abl* interaction and visualized by peroxidase histochemistry.

(A) Dorsal view of a wild-type CNS (late st 13) stained with mAb 22C10. Note combined MP fascicle (thin arrow) and anterior commissure (asterisk).

(B) *N/abl* embryo, staged and oriented as for the wild type (A). Thick arrow indicates a gap in the combined MP fascicle. Pioneer neurons aCC (denoted aC) and dMP2 (labeled d2) are readily identified in the affected hemisegment. Normal-looking anterior commissure in the mutant embryo is indicated by an asterisk.

(C) Early st 13 *N/abl* CNS, showing defects in the initial development of the MP fascicle. A stalled vMP2 growth cone is seen in the second segment from the left; contrast this with the normal appearance of the vMP2 growth cones in the two more posterior segments (arrows; the cell vMP2 is labeled v2).

(D) Late st 13 *N/abl* CNS stained with anti-FasII. Stalled dMP2 growth cone can be seen in the hemisegment on the right (labeled d2); note presence of the glial substratum cell LG5 in this hemisegment. Contrast this mutant segment with the fully developed MP fascicle (thin arrow) in the hemisegment on the left.

(E) Lateral view of the PNS of a st 17 *N/abl* embryo stained with anti-FasII to label motor axons.

(E′) The same segments as (E), but with relevant structures traced on the image. The tracheal lumen is the long dorsal/ventral profile labeled T; the peritracheal cells, which are the direct substratum for the ISN, are visible in the Nomarski image of both segments (circled in [E′] and labeled PT). Note that the ISN stalls and frays in the segment on the left precisely while it is growing along the peritracheal cells; contrast with the segment on the right. Genotypes of *N/abl* embryos in this figure are: N^{ts1};abl^{1} (B), N^{ts1};abl^{1}/abl^{2} ([C] and [D], and N^{55e11}/N^{+};abl^{1}/abl^{2} ([E] and [E′]).

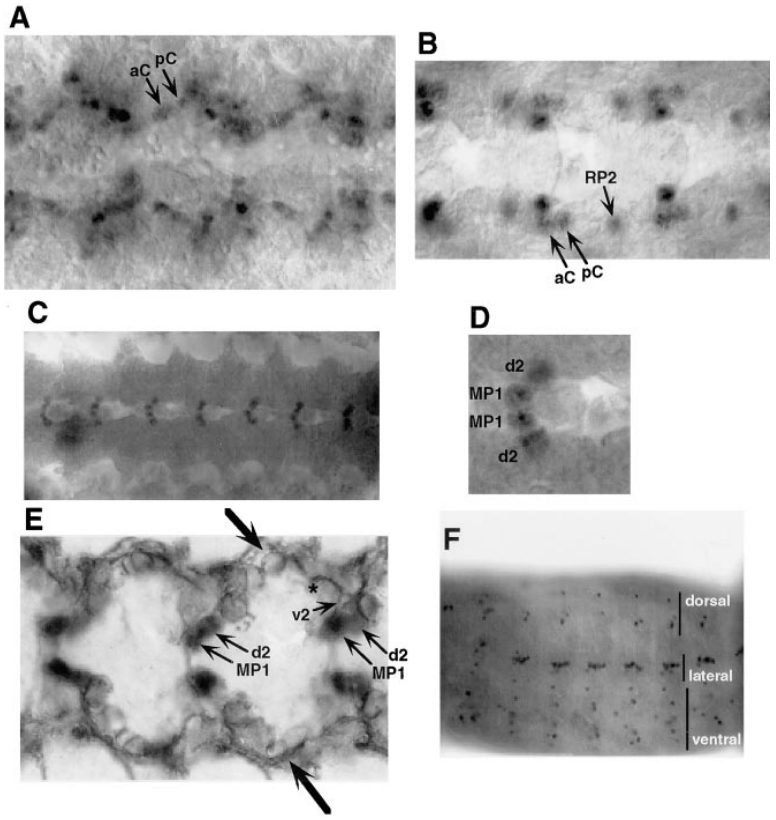

Figure 4. Molecular Analysis of Axonal defects and Cell Identities in *N/abl Embryos*

N/abl embryos were stained with antibodies to label particular cells in the CNS (A–E) and PNS (F) and visualized with peroxidase histochemistry.

(A) Anti-Ftz (st 12/5). Pioneer neurons aCC and pCC are denoted aC and pC, respectively.

(B) Anti-Eve (st 13/14). aCC and pCC are labeled aC and pC, respectively, and RP2 is indicated.

(C) Anti-Odd (st 13).

(D) Higher magnification view of one segment from (C). Pioneer neurons dMP2 (labeled d2) and MP1 are indicated.

(E) St 13 embryo double stained with anti-FasII to visualize the axonal pattern and anti-Odd to assay identities of MP neurons. The thick arrow at the top of the panel shows the position of an interrupted MP fascicle (contrast with the normal MP fascicle highlighted with a thick arrow in the lower half of the same segment). In the hemisegment with a disrupted MP fascicle, the rostral pioneer neurons dMP2 (labeled d2) and MP1 are clearly visible and express Odd appropriately; the caudal pioneer cell vMP2 (labeled v2) is also visible and has properly extinguished expression of Odd. The asterisk indicates the stalled growth cone of vMP2. For this experiment, inclusion of 0.08% $NiCl_2$ in the Dab reaction for the Odd staining, but not for the FasII staining, permits clear discrimination of the two histochemical signals (Ni^{2+} gives the Dab reaction product a black color instead of its usual yellow/brown hue).

(F) Anti-Pros labeling of sense organ glia in the PNS (st 17). Dorsal, lateral, and ventral clusters of sense organs are indicated by bars.

Genotypes of *N/abl* embryos are: N^{ts1};abl^1/abl^2 ([A] through [D]) and N^{ts1};abl^1 ([E] and [F]).

Particularly telling tests of neuronal identity in the CNS of *N/abl* embryos are provided by analysis of Eve expression in the neuron RP2 and Odd protein expression in the pioneers of the MP fascicle. *Notch* controls the identities and projections of RP2 and of the MP2 progeny cells, and the effect of *Notch* on the fates of these cells can be assayed by their expression of Eve and Odd, respectively (Spana and Doe, 1996). These are, however, among the neurons whose axons are also affected by the *Notch/abl* interaction. Thus, if the axonal defects observed in *N/abl* embryos are due to *Notch*-dependent alterations of cell identity, we should be able to detect precisely these alterations by assaying the expression of Eve and Odd.

In wild-type embryos, Eve is expressed in RP2 but not in its sibling cell (RP2*sib*). We found Eve expression to be wild type in RP2 and RP2*sib* in 98% of hemisegments of N^{ts}/*abl* embryos (n = 186 hemisegments; Figure 4B) and >88% of hemisegments of DfN^8/+;*abl*/*abl* embryos (n = 258). At the time that the MP fascicle is pioneered, Odd protein is expressed in the MP1 and dMP2 neurons but not in vMP2 (the sibling cell to dMP2). *Notch* is responsible for differentiating the fates of dMP2 and vMP2. We found that 97% of st 13/14 N^{ts}/*abl* hemisegments had the proper pattern of Odd-expressing cells (n = 337 hemisegments; Figures 4C and 4D). Moreover, upon double staining a *N/abl* embryo with anti-Odd and anti-FasII, we observed appropriate Odd staining even in a hemisegment in which the MP fascicle has failed to develop (Figure 4E). This argues directly against the model that the failure to form the MP fascicle in *N/abl* embryos arises from a *Notch*-dependent transformation in the identities of the dMP2 and vMP2 pioneer neurons. Similarly, the notion that PNS axonal defects in *N/abl* embryos might arise from a *Notch*-dependent interconversion of identity between sensory neurons and their sibling glia is inconsistent with the observation that the peripheral pattern of 22C10 expression (a marker for PNS neurons) and of Pros expression (a marker for sense organ glia; Vaessin et al., 1991) is generally unaffected by the *N/abl* interaction (Figures 2F and 4F).

Genetic Separation of the Functions of *Notch* in Cell Fate Versus Axon Patterning

The experiments above suggest that we cannot account for most of the axonal defects in *Notch/abl* embryos on the basis of observed transformations of pioneer neuron identity. We therefore asked the converse question: whether *Notch*-dependent transformations of pioneer neuron identity are sufficient to produce axonal defects like those observed in *N/abl* embryos. We prepared embryos that were N^{ts1};*elav-GAL4*;*UAS-Notch* and shifted them to restrictive temperature in mid-embryogenesis. In these embryos, the endogenous *Notch* is inactivated by the temperature shift after the completion of neuroblast segregation but during the time when neuronal identities are still being specified and prior to axonogenesis. The GAL4 system (Fischer et al., 1988) then restores

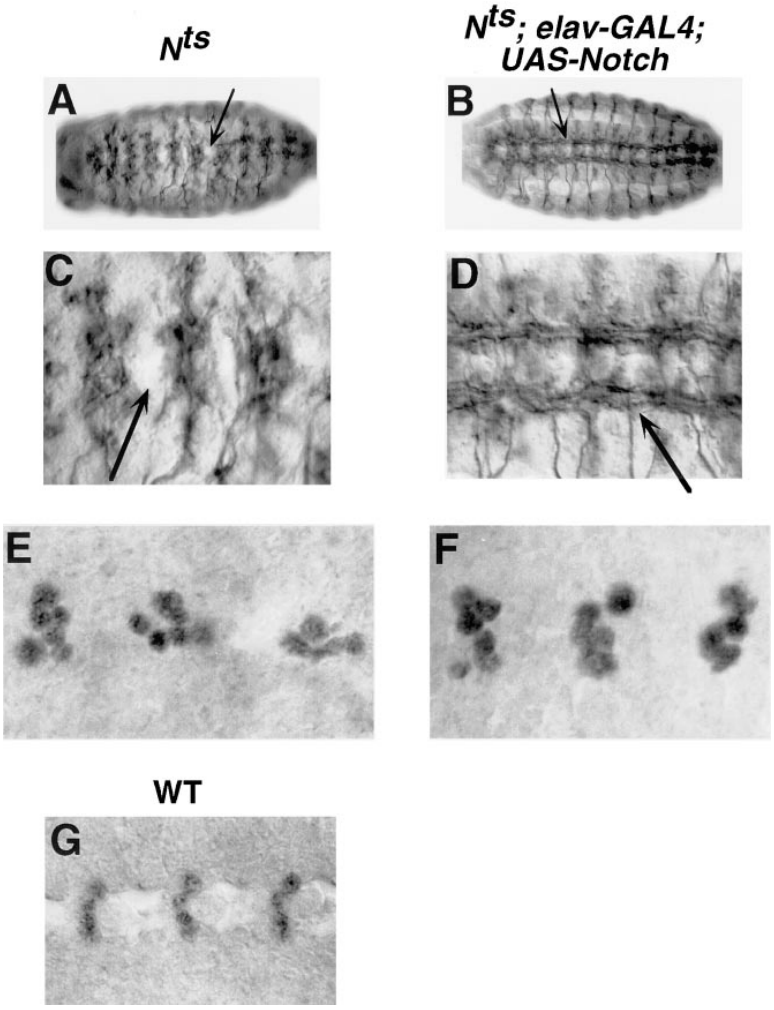

Figure 5. Separation of the Axonal and Cell Fate Functions of *Notch*

Notch^{ts1} embryos that did ([B], [D], and [F]) or did not ([A], [C], and [E]) bear *elav-GAL4* and *UAS-Notch* transgenes were collected at permissive temperature, shifted to restrictive temperature in mid-embryogenesis, and fixed for immunocytochemistry.

(A–D) Ventral views of st 16 embryos stained with anti-FasII. Arrows indicate positions of longitudinal axon tracts (absent in [A] and [C]). (C) and (D) are higher magnification views of the same embryos shown in (A) and (B).

(E–G) Embryos stained with anti-Odd.

(G) A wild-type embryo.

wild-type *Notch* to each neuron at about the time it begins to extend its axon, after its identity has been decided.

As observed previously (Giniger et al., 1993a), we find the characteristic pattern of *Notch*-dependent axonal defects in >90% of *N*^{ts1} embryos subjected to our standard temperature shift protocol (Figures 5A and 5C). In contrast, the axon scaffold of the CNS is rescued to wild type or nearly wild type in >80% of *N*^{ts1};*elav-GAL4*;*UAS-Notch* embryos (n = 102 st 15–17 embryos; Figures 5B and 5D). As assayed by staining with anti-FasII, 49% of embryos showed rescue of longitudinal tracts in all hemisegments and 32% of embryos showed residual defects in just a single hemisegment. In only 19% of cases did *N*^{ts1};*elav-GAL4;UAS-Notch* embryos have CNS axonal aberrations that overlapped in severity those observed in the *N*^{ts} control.

By monitoring the expression of Odd and Eve (Figures 5E–5G), we verified that expression of wild-type *Notch* via *elav-GAL4* does not rescue *Notch*-dependent defects in cell identity. In temperature-shifted *N*^{ts} embryos, we find 5.7 ± 1.6 Odd-positive neurons per neuromere, versus 4 Odd$^+$ cells in wild type (mean ± SD; n = 117 segments). By comparison, the number of Odd$^+$ cells found in *N*^{ts1};*elav-GAL4*;*UAS-Notch* embryos was 5.1 ± 1.2 (n = 135 segments). Analogous results were found for the *Notch*-dependent transformation of RP2*sib*→RP2, as assayed with anti-Eve (data not shown).

These data show directly that the *Notch*-dependent perturbations of cell identity induced in temperature-shifted *N*^{ts} embryos are not sufficient to produce the axonal defects observed in these embryos. They therefore provide strong evidence that the requirement for *Notch* in axon patterning reflects a function of the protein at the time of axon outgrowth, genetically separable from the role of *Notch* in the establishment of cell identity.

Notch Localization in Axons and Growth Cones of Cultured Primary Neurons

Abl is localized to developing axons, and it is thought that Abl works in the axon directly to control cytoskeletal organization and function (Bennett and Hoffmann, 1992). Might Notch also act in the axon to control axon extension directly? Notch is known to be in mature nerves (Fehon et al., 1991), but its presence in developing nerves, and specifically in growth cones, has not been investigated. Since Notch expression in substratum cells interferes with visualizing growth cones in situ, we examined

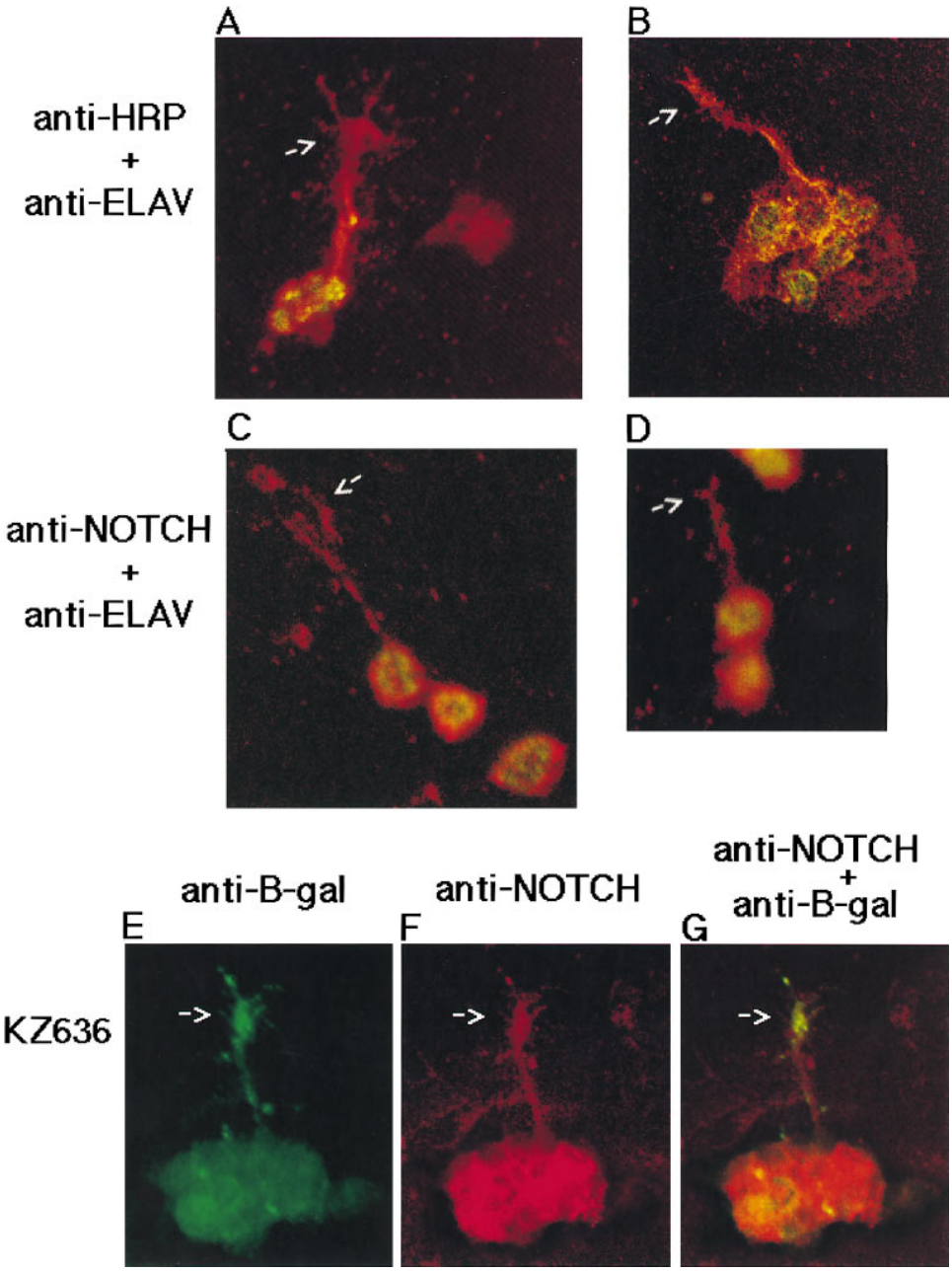

Figure 6. Subcellular Localization of Notch in Cultured Primary *Drosophila* Neurons

Wild-type *Drosophila* embryos were mechanically dissociated, and cells were plated on clean glass coverslips and allowed to develop overnight. Cells were then fixed and processed for indirect immunofluorescence.

(A and B) Cells were double labeled with anti-Elav to label the nuclei of all neurons (FITC, green) and with anti-HRP to visualize neuronal cell morphology (Texas Red). Axons growing directly on glass often terminated in flat, spiked, or bulbous structures that resemble growth cones (white arrows).

(C and D) Cells were double labeled with anti-Elav (FITC, green) and with anti-Notch (Texas Red). Axons growing directly on glass terminated in structures that resembled growth cones and were recognized by anti-Notch antibodies (white arrows).

(E, F, and G) Cells were prepared from embryos that express a kinesin-β-galactosidase fusion in neurons (KZ 636) and were double stained with anti-β-galactosidase (FITC, green) and anti-Notch (Texas Red). Neuronal growth cones (white arrow) identified with the marker kinesin-β-gal were found also to label with anti-Notch. Two unrelated anti-Notch antibodies were used in these experiments. The experiment of (C) and (D) employed a rabbit polyclonal antibody (E. G., unpublished data), while the experiment of (E) through (G) employed the widely used mAb C17.9C6 (Fehon et al., 1991).

the localization of Notch protein in primary *Drosophila* neurons cultured in vitro (Spana and Doe, 1996).

Primary fly embryo neurons were differentiated in culture and analyzed by indirect immunofluorescence with anti-HRP, to characterize neuronal morphologies, or with anti-Notch. All samples were also labeled with anti-Elav (mAb 44C11), to verify that the cells being examined were neurons. Figures 6A and 6B show some typical

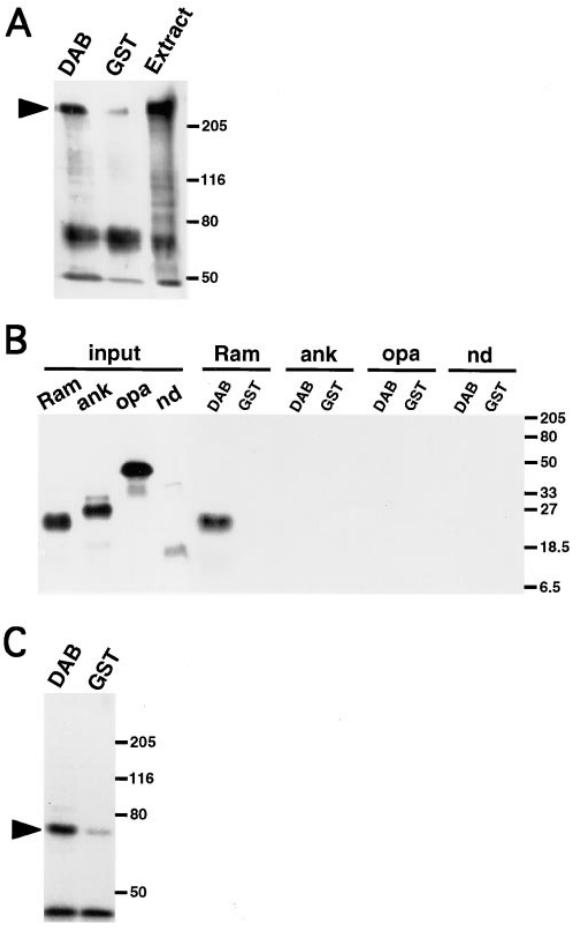

Figure 7. Binding of the Disabled PTB Domain to the Notch Intracellular Domain In Vitro

(A) Western analysis of Disabled binding to wild-type Notch in a total *Drosophila* embryo extract. Crude embryo extract was incubated with glutathione beads bearing either a fusion of the Disabled PTB domain to GST or else GST alone. Beads were washed and boiled in Laemmli buffer, and associated proteins were analyzed by PAGE and Western blotting with anti-Notch antibodies. Gel lanes: Dab indicates material bound to the Disabled PTB domain, GST indicates material found in association with GST, and Extract shows the pattern of immunoreactive bands found in the starting embryo extract. Large arrowhead indicates full-length Notch protein (~300 kDa).

(B) Autoradiograph of a gel analyzing the binding of the Disabled PTB to various functional domains of Notch. Four fragments from the Notch intracellular domain were expressed by in vitro translation and assayed for binding to beads bearing either GST–Disabled PTB or GST alone. Notch domains tested were the Ram23 region (Ram), ankyrin repeats (ank), PEST/OPA region (opa) and notchoid region (nd); see text for details. The left four lanes (input) show the crude material from the in vitro translation reactions. The right eight lanes are arranged as pairs, showing material bound to GST–Dab (indicated as Dab) or GST alone (indicated as GST) for each of the four protein fragments. Binding was observed only for the Ram23 fragment incubated with the Disabled PTB domain.

(C) Autoradiograph of a gel assaying the binding of the purified Notch intracellular domain to the purified Disabled PTB domain in vitro. The N-terminal half of the Notch intracellular domain was expressed in *E. coli*, purified, kinase labeled with ^{32}P, and incubated with beads bearing either GST–Disabled PTB or GST alone. The beads were washed, eluted, and analyzed by PAGE and autoradiography. Dab indicates protein bound to the Disabled PTB; GST indicates material bound by GST alone. Large arrowhead indicates the position of the major purified Notch fragment (M_r, ~70 kDa for the phosphorylated species, including vector sequences and tags). The labeled band at ~45 kDa is an anonymous bacterial protein that copurifies with the Notch fragment and associates nonspecifically with beads.

In each panel, positions of molecular weight markers are indicated by numbers to the right of the gel.

morphologies of cultured fly neurons, as visualized with anti-HRP. These may be compared to Figures 6C and 6D, which show cells labeled with anti-Notch. Notch protein was clearly detected on the entire cell surface, including extending axons, and on a variety of bulbous, spiked, and flattened structures at the tips of axons, which have the appearance of growth cones.

To test further whether the Notch-containing structures at the ends of axons were bona fide growth cones, we double labeled cell preparations for Notch and for a known growth cone marker, kinesin-β-galactosidase (Giniger et al., 1993b). Figure 6E shows a cluster of neurons in which a growth cone can be identified by its high level of β-gal immunoreactivity. Figures 6F and 6G show that the same structure also labels with anti-Notch, verifying that Notch protein is present on the growth cones of axons extending in culture.

Notch Binds Disabled In Vitro

What might be the physical basis of the *N/abl* genetic interaction? It is unlikely that the absence of Abl is affecting Notch protein levels, since Western analysis of extracts from homozygous *abl*$^-$ females detects wild-type amounts of Notch protein (normalized for total protein; data not shown). Moreover, such a mechanism would be expected to alter *Notch*-dependent cell identities as well as cell morphologies, and we have shown above that this does not generally occur. We considered whether Abl might bind Notch directly, but again this seems unlikely. While Abl contains a variety of protein interaction domains (Wang, 1993), Notch does not resemble its known ligands. It has recently been shown that the *Drosophila* Numb protein includes a PTB domain that binds two sites in the intracellular domain of Notch, even when Notch is not phosphorylated (Guo et al., 1996). Recalling that the *abl*-interacting gene *disabled* includes a PTB domain closely related to the Numb PTB, and which like Numb can bind to nonphosphorylated targets (Howell et al., 1997), we tested whether Dab could bind the intracellular domain of Notch in vitro.

Three experiments demonstrate that the PTB domain of *Drosophila* Disabled binds directly to the intracellular domain of Notch in vitro. First, beads bearing a glutathione S-transferase (GST) fusion of the Dab PTB domain were incubated in an extract of total embryo protein. Western analysis of the protein bound by Dab shows that GST–Dab selects Notch protein out of an embryo lysate, whereas GST alone binds only a small amount of Notch nonspecifically (Figure 7A).

We next asked what portion of Notch was recognized by Dab. We expressed four protein fragments, each of which represented a distinct functional domain from the intracellular tail of Notch. These were the RAM23 region (amino acids 1766–1896), the ankyrin repeats (amino acids 1896–2109), the PEST/OPA region (amino acids 2262–2606), and the notchoid region (amino acids 2612–

2703). The four proteins were translated in vitro in reticulocyte lysates and assayed for binding to GST–Dab as above. Of the four Notch domains, only the RAM23 peptide bound to GST–Dab, while none of the four bound to GST alone (Figure 7B). This pattern is similar but not identical to the pattern of Notch binding to the Numb PTB: like Dab, the Numb PTB binds to the Notch RAM23 domain but not to the ankyrin repeats or PEST/OPA region. Unlike Dab, Numb does bind to the notchoid domain.

Finally, to determine whether the Dab–Notch interaction is direct, we purified a stable and soluble N-terminal fragment of the Notch intracellular domain (amino acids 1767–2235) from bacteria and assayed its binding to the purified Dab PTB domain. Figure 7C shows that the Notch intracellular domain is precipitated by GST–Dab beads but not by GST alone, demonstrating that the purified Dab PTB domain can bind directly to purified Notch intracellular domain in vitro.

Genetic Interaction of *Notch*, *abl*, and *disabled*

The data above demonstrate that *Notch* interacts genetically with *abl* and biochemically with Disabled. These results beg the question whether *Notch* interacts genetically with *disabled*. Since we do not have isolated *dab* alleles, we cannot test directly their genetic interactions with *Notch*. We can, however, ask whether flies that are triply heterozygous for all three mutations, *Notch, abl*, and *dab*, display any synthetic phenotypes.

We constructed flies that were both heterozygous for a strong *Notch* allele (N^{8} or N^{55e11}) and for one of two unrelated chromosomes that bear strong mutations of both *abl* and *dab* ($abl^{1}dab^{M54}$ and $Df(3L)std^{11}$). All four pairwise combinations caused defects in eye development, giving rise to flies with rough eyes reminiscent of the defective eyes observed in *abl* homozygotes. For the strongest allelic combination, $N^{8}/+;abl^{1}dab^{M54}/+$, the penetrance of eye defects was 100%, whereas even the weaker combinations using N^{55e11} gave a penetrance of 78% and 86%, respectively, in a typical experiment. Rough eyes were not observed in either *abl/dab* double mutants alone or in $<10\%$ of $N^{-}/+;abl^{1}/+$ double heterozygotes.

We also examined the axonal phenotype of embryos that were $N^{ts};abl^{-/-};dab^{-/+}$ at various temperatures, to ask whether we could discern synergistic axonal defects from the conjunction of all three mutations. These experiments were inconclusive, however; while such embryos showed more severe defects than did embryos that were either $N^{ts};abl^{-/-}$ or $abl^{-/-};dab^{-/+}$, it was not clear whether the phenotypes observed were more severe than those expected from the summed effects of the constituent mutant interactions (E. G., unpublished data).

Discussion

Our data implicate the Abl tyrosine kinase in signaling by the receptor Notch in particular developmental contexts. *Notch* and *abl* mutations display a synergistic genetic interaction, with appropriate combinations of *Notch* and *abl* alleles causing synthetic lethality and defects in axon extension. Analysis of substratum cells and pioneer neurons for affected axon tracts fail to identify alterations in cell identity or differentiation that adequately explain the observed axonal defects, and thus raises the possibility that the *N/abl* interaction may somehow disturb axonal morphogenesis directly. Consistent with this idea, we find that the requirement for *Notch* in axon patterning is genetically separable from its role in control of cell identity. Moreover, we find that Notch protein, like Abl and Dab, is localized to developing axons and that Notch is present in growth cones. Finally, our data suggest a possible biochemical basis for the genetic interaction of *Notch* and *abl*: the Abl accessory protein, Disabled, binds directly to the intracellular domain of Notch in vitro, and *dab* mutations interact genetically with *abl* and *Notch* mutations in vivo.

Several lines of evidence argue that the *Notch/abl* interaction reveals the existence of a novel signaling pathway. First, the developmental defects observed in *N/abl* embryos are restricted to a process that is known to require each of these proteins separately: formation of particular axonal segments in the central and peripheral nervous system of the embryo. Processes that require *Notch* but not *abl*, such as segregation of neural precursors, are unaffected by the genetic interaction, as are processes that require *abl* but not *Notch*, such as extension of commissural axons (Elkins et al., 1990). Second, generalized developmental defects are not observed in cell types that employ both of these proteins for unrelated purposes. In muscle development, *Notch* is required for specification of myoblast identity (Corbin et al., 1991) and *abl* for maintenance of muscle attachments (Bennett and Hoffmann, 1992), yet muscle development appears largely normal in *N/abl* embryos. Thus, the presence of severe axonal defects in *N/abl* embryos suggests a functional interaction between these proteins.

How might Notch and Abl collaborate during axon extension? In particular, is it plausible that Notch should, in some circumstances, act in the growth cone to control axon extension directly? Previous work demonstrated that Notch and its ligand, Delta, are required for the development of a specific subset of axons in the embryonic nervous system (Giniger et al., 1993a), but it was not possible to distinguish whether this reflected a direct role for Notch in axons or simply a secondary consequence of effects on cell identities. In the current experiments, we show that *Notch*-dependent errors in axon patterning can occur without significant alterations of cell identity (in the context of *abl*), and conversely, we show that *Notch*-dependent alterations of cell identity can occur without concomitant errors in axon patterning (so long as we provide wild-type *Notch* at the time of axon outgrowth). The demonstration that changes in cell fate are neither necessary nor sufficient to produce the characteristic pattern of *Notch*-dependent defects in axon extension is difficult to reconcile with the model that all such axonal defects are secondary to changes in cell identity. They are more readily explained by the model that Notch and Abl both work directly in the axon to control the development of affected nerves. A caveat to the interpretation of the *N/abl* experiment, of course, is that we cannot formally rule out the possibility that

small changes in the level of marker gene expression that do not alter the apparent pattern of expressing versus nonexpressing cells could nonetheless be sufficient to alter cell properties.

Data consistent with the model of a direct function for Notch in axons have recently been published by Rubin, Goodman, and coworkers. They demonstrated that an upstream element of the *Notch* pathway, the gene *kuzbanian*, is required in the mature neuron for axon extension (Fambrough et al., 1996; Rooke et al., 1996; Pan and Rubin, 1997). Given the requirement for Kuz in Notch biosynthesis, and the observation that axonal defects in *kuz* mutants are largely the same as those caused by *Notch*, the simplest interpretation of these data is that *kuz* affects axons by controlling Notch levels. Importantly, it was shown that the effect of *kuz* on axons is not likely to be secondary to changes in cell fate (Fambrough et al., 1996; Pan and Rubin, 1997).

While our data implicate Abl in the control of axon extension by Notch, they do not exclude the possibility that Abl also contributes somewhat to the control of cell identity by Notch in some circumstances. The data we report analyzing the expression of cell-specific molecular markers in *N/abl* embryos do reveal a low but consistent level of defects in cell identity. Perhaps Abl plays a minor or redundant role in other aspects of Notch function, even within the *numb* or *Su(H)* pathways. It may well be that this effect contributes somewhat to the axonal defects we observe.

There are a variety of molecular models that could account for the genetic interaction of *Notch* and *abl*. For example, it may be that Delta and Notch lie in one pathway, presumably including Disabled, while Abl acts in a parallel pathway downstream of some unidentified receptor. However, given the genetic and biochemical evidence for Abl–Dab interaction (Gertler et al., 1993; Howell et al., 1997), it is attractive to speculate that Dab may act as an adaptor protein that links Notch to Abl in response to a signal from Delta (Figure 8). We imagine that recruitment of Abl by Notch in turn engages the actin cytoskeleton via mechanisms similar to those that have been studied in vertebrate systems (Pendergast et al., 1991; Hall, 1992; Ridley et al., 1993). The notion that Notch may use distinct signaling pathways to control different downstream events—Su(H) to control cell identity and Abl to control cell morphology—is consistent with analysis of other signaling receptors. For example, receptor tyrosine kinases typically bind and activate a complex array of intracellular signaling proteins upon ligand induction (Schlessinger and Ullrich, 1992), and different downstream signaling pathways are often responsible for different aspects of the induced phenotype (Kazlauskis et al., 1992; Fukunaga et al., 1993). Finally, there is extensive precedent for receptors that control cell fate in some developmental contexts and cell motility or axon extension in others (Hirata et al., 1993; Ebens et al., 1996).

Recent work has identified a large number of proteins that are implicated in axon growth and guidance in vivo (reviewed by Goodman and Shatz, 1993; Tessier-Lavigne and Goodman, 1996). These include receptors such as UNC5 and Eph family tyrosine kinases, as well as signaling proteins such as Dock and Rac1. It has

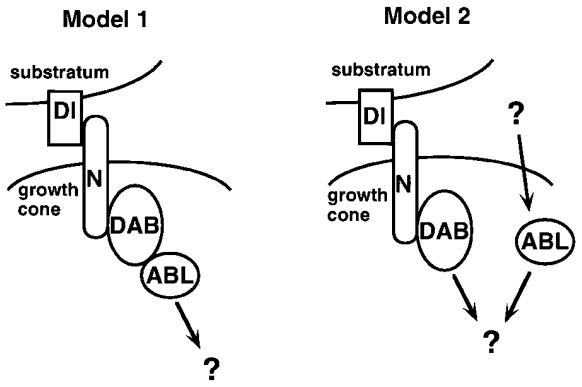

Figure 8. Models for the interaction of Notch with Abl and Disabled

Notch is indicated as N, the Notch ligand Delta is labeled as Dl, and Disabled is labeled as Dab. The targets of Abl (and of Dab in Model 2) are unknown and are shown as a question mark, though by analogy to work from vertebrate systems it is plausible that Abl recruits Rho family GTPases, which would in turn be expected to play a fairly direct role in actin assembly and myosin activity. Two simple models are shown; many other models remain possible.

(Model 1) The data are consistent with Disabled acting as an adaptor protein that recruits Abl to the intracellular domain of Notch in response to a signal from substratum-bound Delta protein.

(Model 2) We cannot exclude the possibility that Abl acts in parallel to Disabled, downstream of some unidentified intra- or extracellular signal.

been very difficult, however, to link these proteins together into biochemical pathways and harder still to implicate such pathways genetically in characterized guidance decisions in vivo. We have shown previously that Delta is expressed by a required substratum for the ISN, and that *Delta* and *Notch* are both required for proper guidance along this substratum (Giniger et al., 1993a). We have now shown that *Notch* interacts genetically with the *abl* kinase and biochemically with the Abl accessory protein Disabled, both of which are required in the axon for the extension of many if not most axons. Thus, this is one of the only systems where genetic and biochemical data converge to suggest a direct link between a transmembrane receptor that is genetically required for an identified axon guidance decision in vivo and a known axonal signaling protein.

Our evidence for interaction between Notch and Abl in *Drosophila* immediately raises the question whether these two highly conserved proteins collaborate in other biological contexts. Abl has been studied for nearly 20 years, as it was one of the first oncogenes to be convincingly linked to the etiology of a common cancer: *abl* is mutated in 90% of chronic myelogenous leukemia (CML) and in Ph^+ acute lymphocytic leukemia (ALL) (Goff et al., 1980; Fainstein et al., 1987). In mammals, *abl* is expressed in normal as well as neoplastic lymphoid cells and is required for normal hematopoietic development (Schwartzberg et al., 1991; Tybulewicz et al., 1991). It has been exceedingly difficult, however, to identify the remainder of the *abl* signaling pathway. In particular, vertebrate Abl has yet to be linked definitively to any receptor or extracellular ligand in vivo, though it has been associated with integrin function in vitro (Lewis et al., 1996). We note that *Notch*, like *abl*, is implicated in

mammalian hematopoiesis, specifically lymphoid development (Milner et al., 1996; Robey et al., 1996), and like *abl* gives rise to lymphoid tumors when activated inappropriately (Ellisen et al., 1991). Perhaps Abl is an element of Notch signaling in humans, as we have shown in flies. If so, it may be that CML and ALL are human diseases of the *Notch/abl* signaling pathway.

Experimental Procedures

Drosophila Stocks

abl^{1}, *abl*^{P}, and *Df(3L)st*^{i7} were obtained from Corey Goodman. P[*adh*$^{+}$;*abl*$^{+}$], *abl*^{1}*dab*^{M54}, and *Df(3L)std*^{11} were obtained from Frank Gertler and Mike Hoffman. All *abl* chromosomes were cleaned by recombination prior to use. *Df(1)N*^{8}, *N*^{55e11}, *N*^{ts1}, *Dp(w*$^{-}$*N*$^{+}$)·*Y*, *elav-GAL4*, and *lacZ*-marked balancers for the first and third chromosomes were obtained from Y. N. Jan. *N*^{ts1} was isogenized prior to use. All mutations are described in Lindsley and Zimm (1992). *UAS-Notch* was obtained from Gerold Schubiger. All quantitation of penetrance and expressivity employed heteroallelic combinations of *abl* alleles. In quantitating embryonic phenotypes, we found that for all allelic combinations, a fraction of both *abl* heterozygous and homozygous embryos displayed nonspecific disruption of embryo morphology; such animals were not included in the quantitative analysis.

The protocol for temperature-shift experiments was as follows: embryos were collected at 18°C for 6 hr, aged an additional 6 hr at 18°C, shifted to 32°C for 7.5 hr, and then fixed. This timing was designed to produce highly penetrant axonal phenotypes while minimizing neurogenic defects, taking into account the phenotypic lag between the time of the temperature shift and the decay of activity of *N*^{ts1} mutant protein (Hartenstein and Posakony, 1990; Giniger et al., 1993a).

Embryo Immunocytochemistry

Embryos were collected, fixed, and stained by standard methods (Bodmer and Jan, 1987). Detection was typically carried out with peroxidase-conjugated secondary and tertiary reagents developed with DAB. Embryo genotypes were established by detection of a *lacZ*-marked balancer using anti-β-galactosidase antibodies. In some experiments, detection of anti-β-gal was done with an alkaline phosphatase–conjugated secondary antibody developed with X-phosphate. For experiments conducted in this manner, all embryo incubations and washes were in 100 mM Na Phosphate (pH 7.2) with 0.1% Tween 20, and the peroxidase reaction was performed prior to the alkaline phosphatase reaction. When α-Odd was used, embryos were first incubated for 15 min in 9:1::methanol:30% H_2O_2, washed, and then blocked. Staging of embryos was by the criteria of Campos-Ortega and Hartenstein (1985).

Samples were dehydrated with ethanol and whole mounted in Permount or in JB4 plastic embedding media (Polysciences), or they were filleted, essentially by the method of Desai and Zinn (The Fly Motor Axon Home Page, http://www.caltech.edu/~zinn/motoraxons/fma%20home %20page.html). Filleted embryos were mounted in 90% glycerol. Embryos were examined under Nomarski optics and photographed on Ilford XP2 film. Where necessary, multiple focal planes were montaged digitally, by scanning negatives onto a Photo CD and combining images in Photoshop.

Antibodies

The antibodies used were as follows: Rb α-HRP, 1:100 after affinity purification (Cappel); Rb α-β-gal, 1:10,000 (Cappel); M α-β-gal, 1:750 (Boehringer Mannheim); mAb 22C10, 1:50 when used with a POD-conjugated 2° or 1:375 when used with a biotinylated 2°, a gift from Seymour Benzer; mAb 1D4 (anti-FasII), 1: 350, from Greg Helt and Corey Goodman; mAb 3C10 (anti-Eve), 1:2, from Corey Goodman; mAb 44C11 (anti-Elav), 1:10, from Y. N. Jan; Rb anti-Prospero, 1:1,000, from Harald Vaessin; M α-Ftz, 1:1,000, from Ian Duncan and Dianne Mattson-Duncan; Rb α-myosin heavy chain, 1:500, from Dan Kiehart; mAb C17.9C6 (α-Notch ascites), 1:1,000, from Robert Mann and Spyros Artavanis-Tsakonas; Rb α-Notch, 1:750 (immunocytochemistry) or 1:3,000 (Western blotting), E. G., unpublished data; and Rb α-Odd, 1:6,000–1:20,0000, from Doug Coulter and Ellen Ward. Peroxidase-coupled, biotinylated, and fluorescent secondary antibodies were from Jackson Immunologicals; alkaline phosphatase–conjugated secondaries were from Fisher; and biotinylated secondary antibodies were detected using the Vectastain Elite tertiary (Vector Labs).

Primary Culture of *Drosophila* Cells

In vitro culture of primary *Drosophila* neurons was done as decribed by Spana and Doe (1996). Samples were mounted in FluoroGuard (BioRad) and examined with a BioRad MRC600 confocal microscope. Authenticity of the fluorescent signals was verified by omitting or replacing the primary antibody, and by separate, sequential excitation of the two fluorophores in double-label experiments.

Cloning and Expression of Notch and Disabled Fragments

Expression of Notch Intracellular Domain in Bacteria

Phage containing *Notch* cDNAs were isolated from the Kauvar E7 library by hybridization. Sequences encoding the complete intracellular domain of Notch were amplified by PCR and cloned between the BglII and KpnI sites of the His_6 vector pRSET A (Invitrogen), after first introducing two copies of a protein kinase A recognition sequence into the BamH1 site of the vector. One isolate (pEG203) acquired an adventitious frameshift mutation at nt 7448, appending the amino acid sequence RPPT, followed by a stop codon, to residue K2235; this isolate made an abundant, stable and soluble fragment of Notch and one that appears to have complete *Notch* signaling activity in vivo (Doherty et al., 1996; Larkin et al., 1996; E. G., unpublished data). All cloning steps were performed in DH10B (Gibco/BRL) or BL21 (Novagen).

BL21::λDE3::pLysS::pEG203 cells were grown at 37°C to mid-log phase in 100 ml LB containing carbenicillin and chloramphenicol. IPTG was added to 1 mM, after which cells were grown 4 hr and then harvested. Induced cells were resuspended in 10 ml of lysis buffer, containing 8 M urea, 50 mM Na Phosphate (pH 7.2), and 100 mM NaCl plus protease inhibitors (a 1:100 dilution of leupeptin, 1 mg/ml; pepstatin, 1 mg/ml; aprotinin, 2 mg/ml; benzamidine, 10 mg/ml; PMSF, 100 mM; in DMSO). After sonication, 1.5 ml Ni^{2+} resin (Invitrogen) was added to the lysate and allowed to bind for 2 hr with rocking at RT. Bound protein was washed twice with lysis buffer, twice with lysis buffer containing 1 M NaCl, and twice more with lysis buffer. Protein was renatured by dialysis into 25 mM HEPES (pH 7.5), 100 mM NaCl, 0.5% NP40, 2 mM β-mercaptoethanol, and 0.1 mM PMSF. To kinase label the purified Notch, 100 μl of Notch-bearing beads were washed into kinase buffer (20 mM Tris [pH 7.6], 100 mM NaCl, 12 mM $MgCl_2$, and 1 mM DTT) and then incubated in a 250 μl reaction volume of kinase buffer containing 50 μCi [γ-^{32}P]ATP and 50 U heart muscle kinase (Sigma) at 37°C for 1 hr. Beads were washed in Notch renaturation buffer (above), then eluted with renaturation buffer containing 20 mM EDTA.

Subcloning and Expression of Fragments of the Notch Intracellular Domain

Four nonoverlapping fragments of the Notch intracellular domain were PCR amplified and subcloned between the NheI and BglII sites of pRSET A. The Notch fragments were: Ram23 region, nucleotides 6034–6419; ankyrin repeats, nucleotides 6417–7065; PEST/OPA region, nnucleotides 7524–8559; and notchoid region, nucleotides 8572–8856 (numbering as in Wharton et al., 1985). In each case, a stop codon was introduced after the final amino acid. Notch fragments were expressed in a rabbit reticulocyte lysate transcription/translation system (TNT, Promega).

Subcloning and Expression of the Disabled PTB Domain

Nucleotides 1176–1619, corresponding to amino acids K36–I184 of Disabled, were amplified by PCR and subcloned between the BamHI and EcoRI sites of the GST vector pGEX-2T. GST–Disabled or the parent vector were transformed into BSJ72, and 100 ml cultures were grown to mid-late log phase at 37°C. Cultures were induced with 0.5 mM IPTG, grown an additional 4 hr, and harvested. Cell pellets were suspended in 5 ml lysis buffer (50 mM Tris [pH 7.9], 0.5% Triton X-100, and 1 mM PMSF) at 0°C and lysed by sonication. Extract was cleared by centrifugation (10 Krpm, 10 min), and supernatant was added to preblocked glutathione sepharose beads

(Pharmacia) and rocked for 10 min at RT. Beads bearing GST–Dab or GST were collected by brief centrifugation, washed, and stored at 4°C.

Protein Binding Experiments

Binding of Dab to Notch ICD Purified from Bacteria

Labeled, purified Notch intracellular domain was added to binding buffer (25 mM HEPES [pH 7.5], 50 mM NaCl, 1% NP40, 0.1% SDS, and 15 mM β-mercaptoethanol) containing 2% BSA, incubated with blocked GST beads for 30 min at 4°C at a ratio of 10 μl beads per 750 μl diluted Notch protein, and cleared by centrifugation (17 Krpm, 10 min). To each cleared sample, 10 μl of blocked GST or GST–Dab beads were then added and incubated with rocking for 90 min at 4°C. Beads were pelleted by brief centrifugation, washed five times with 500 μl of binding buffer, resuspended in 20 μl Laemmli sample buffer, and boiled. Protein was separated in a 7.5% SDS-polyacrylamide gel, the gel was dried down, and the Notch protein was visualized by autoradiography.

Binding of Dab to Fragments of the Notch ICD Translated In Vitro

Protocol for binding experiments employing in vitro translated Notch protein was essentially the same as for purified Notch, except that the binding buffer contained 0.5% NP40 and no SDS. Bound proteins were separated on a 12% SDS-polyacrylamide gel. The gel was fixed, soaked in Entensify (NEN), dried down, and visualized by autoradiography.

Binding of Dab to Notch in a Crude Embryo Lysate

Total embryo lysate was prepared as follows. Zero to twenty-four hour embryos were harvested, dechorionated with 50% bleach, washed with 0.7% NaCl and 0.3% Triton X-100, washed once with water, and transferred to an ice-cold Dounce homogenizer. Embryos were then washed once with 0°C LB (25 mM Hepes [pH 7.5], 300 mM NaCl, 0.5% NP40, 15 mM β-mercaptoethanol, 5 mM NaF, 10 mM Na Pyrophosphate, and 25 mM β-glycerophosphate). Embryos were suspended in three embryo volumes of lysis buffer containing a 1:100 dilution of protease inhibitors (see above) and homogenized on ice by 15 strokes with an A pestle, followed by 20 strokes with a B pestle. Embryo homogenate was incubated with gentle rocking for 1 hr at 4°C and cleared by centrifugation (17 Krpm, 30 min). Cleared supernatant was harvested, and GST beads were added at a ratio of 30 μl beads per 500 μl extract. The supernatant was then rocked for 45 min and again cleared by centrifugation (17 Krpm, 20 min). Extract supernatant was then diluted (400 μl → 1 ml) with lysis buffer lacking NaCl (Buffer A) to reduce the NaCl to 125 mM. Preblocked GST–Dab or GST beads were added to the cleared lysate at a ratio of 30 μl beads per 1 ml diluted extract and incubated overnight at 4°C with gentle rocking. Beads were collected by brief centrifugation and washed six times with Buffer A containing 50 mM NaCl and 0.1 mM PMSF. Beads were resuspended in 30 μl Laemmli sample buffer and boiled, and proteins were electrophoresed through a 6% SDS-polyacrylamide gel. Gel was transferred to nitrocellulose, probed with anti-Notch antibodies and peroxidase-coupled secondary antibody by standard methods, and visualised by chemiluminescence using the Renaissance reagent (NEN).

Acknowledgments

Numerous people have provided me with invaluable advice through the lengthy gestation of these experiments. First, for much indispensable advice about *abl* and its associated genes, I would like to thank Mike Hoffmann, Allen Comer, and particularly Frank Gertler. For advice on genetic interactions, Ed Grell (who also assisted me in cleaning the *abl* chromosomes), Susan Younger-Shepherd, and Harald Vaessin. For advice on the Notch–Disabled biochemical experiments, Kristin Carlberg, Brian Howell, and Mario Lioubin. For advice about assaying and recognizing CNS cell identities, Chris Doe, Roger Jacobs, David Merritt, and Paul Whitington. For unpublished Disabled information and reagents, Jon Cooper and Brian Howell. For advice about the primary cell culture experiments, and for sharing unpublished information about the effects of *Notch* on CNS cell identities, Chris Doe and Jim Skeath. For assistance with some of the embryo fixations, Dan Crowner. For helpful advice concerning all aspects of these experiments, members of my lab, particularly Lisa Connell-Crowley, Sal Fuerstenberg, and Knut Madden. For comments on the manuscript, Lisa Connell-Crowley, Chris Doe, Elaine Ostrander, Jim Priess, and Tom Reh.

I wish to thank the following people for sharing reagents. For antibodies, Seymour Benzer, Ian Duncan, and Dianne Mattson-Duncan; Dan Kiehart, Greg Helt, and Corey Goodman; Robert Mann and Spyros Artavanis-Tsakonis; Harald Vaessin; and Doug Coulter and Ellen Ward. For fly stocks, Susan Younger-Shepherd and Y. N. Jan; Frank Gertler and Mike Hoffman; Gerold Schubiger; and Corey Goodman.

These experiments were supported by a Basil O'Connor Award from the March of Dimes Birth Defects Foundation (#5-FY96–0560), a grant from the U. S. Army Breast Cancer Research Program (#DAMD17–94-J-4266; the conclusions presented do not necessarily reflect the position or policy of the U. S. government and no official endorsement of these results should be inferred), and institutional funds of the FHCRC.

Received November 11, 1997; revised February 2, 1998.

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